AN *IN VITRO* SKIN EQUIVALENT FOR EVALUATION OF SKIN ABSORPTION OF COMPOUNDS

Priya Batheja[1], Prafulla Chandra[2], Vishwas Rai[1] and Bozena Michniak*[3]
[1] *Graduate Student and Research Assistant, Ernest Mario School of Pharmacy, Rutgers – The State University of New Jersey, Piscataway, NJ, 08854*
[2] *Post-Doctoral Associate, New Jersey Center for Biomaterials, Piscataway, NJ, 08854*
[3] *Associate Professor, Ernest Mario School of Pharmacy, Rutgers – The State University of New Jersey, Piscataway, NJ, 08854*

ABSTRACT

Skin Equivalents (SE) or Human Skin Equivalents (HSEs) are skin substitutes that can serve as models for testing the skin permeability of agents from formulations, or for evaluation of formulations themselves on the skin. We have developed a collagen based HSE and HSE containing electrospun poly(DTE carbonate) polymer scaffolds in our laboratory. The culture of these full thickness skin equivalents has been optimized by modification of the culture media and conditions required for growth in order to mimic the barrier properties of human skin *in vivo*. The HSE has been characterized for morphology, lipid composition and barrier properties and compared to a commercially available skin equivalent, and shows similar permeability to a wide range of agents. Skin derived cells were found to populate and proliferate in the electrospun scaffold, which imparts structural stability to the collagen based HSE model. Use of co-cultures of human dermal fibroblasts and human keratinocytes, and conditions such as addition of ascorbic acid are being used to look at effects on morphogenesis and barrier properties of the epidermal layer in these HSE models. Once developed, these skin equivalents can serve as effective models for determination of the permeation of chemical warfare agents (CWAs) or their mimics or the barrier properties of creams such as SERPACWA (Skin Exposure Reduction Paste Against Chemical Warfare Agents).

1. INTRODUCTION

The skin serves as a convenient route for the delivery of agents transdermally for systemic absorption or topically for effects on the skin surface or for action in different skin layers or skin appendages. Permeation or toxicity testing of agents from various transdermal or topical formulations has been carried out on animal models over the years, which differ in their barrier properties from human skin and are facing increasing restrictions for testing due to safety concerns. Human cadaver skin has been extensively used for *in vitro* permeability testing, but is expensive, difficult to procure and demonstrates high variability in permeation data when obtained from different donors and different sites of the body.

Full-thickness skin models or Skin Equivalents (SEs) have been developed by various groups and can be used for skin replacement in burns or as a model for rapid screening of formulations, toxicity testing and for studying the biological properties of skin (Ponec, M. 2002). Today, SEs are available commercially, and some of the most commonly used model are Epiderm® (epidermis only) and Epiderm FT® (Full thickness SE) (MatTek Corporation, Ashland MA, USA) (Bernard, F.-X., Barrault, C. et al. 2000;Boelsma, E., Gibbs, S. et al. 2000). These models, though consistent in permeability and biological responsiveness, have a limitation of possessing weak barrier function and thereby overestimate the permeability of agents through skin (Asbill, C., Kim, N. et al. 2000). This compromised barrier function may be a result of impaired desquamation (Vicanová, J., Boelsma, E. et al. 1998), abnormal skin lipid profiles or the presence of unkeratinized microscopic foci (Mak, V. H. W., Cumpstone, M. B. et al. 1991).

Our group has developed a full-thickness Human Skin Equivalent model (HSE) that is essentially living skin that is grown *in vitro*. The full thickness model has a fully differentiated epidermis (including a stratum corneum) grown on a dermal base made from collagen and fibroblasts. The HSE is grown under modified culture conditions and media with an aim to increase the barrier properties to simulate the barrier properties of skin *in vivo*. The model has been characterized for lipid composition, morphology and permeability to various model agents spanning a range of lipophilicities. Permeability profiles from the HSE have been compared to those of Epiderm FT®. Polymeric nanofibers produced using a technique called electrospinning are being widely used to create tissue engineering scaffolds that more closely mimic natural extracellular matrix (Ma, Z., Kotaki, M. et al. 2005;Smith, L. A. and Ma, P. X. 2004) and are now being considered for generating full thickness human skin models (Ng, K. W., Tham, W. et al. 2005). It has also been demonstrated that using electrospun polymer mats as dermal scaffolding materials helps the skin cells to organize properly, resulting in a well formed tissue (Sun, T., Mai, S. et al. 2005). We are using electrospun fiber

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DEC 2008	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
An In Vitro Skin Equivalent For Evaluation Of Skin Absorption Of Compounds	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Graduate Student and Research Assistant, Ernest Mario School of Pharmacy, Rutgers The State University of New Jersey, Piscataway, NJ, 08854**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002187. Proceedings of the Army Science Conference (26th) Held in Orlando, Florida on 1-4 December 2008, The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**8**	

mats made from the polymer poly(DTE carbonate) (a biocompatible polymer derived from the amino acid L-tyrosine (Bourke, S. L. and Kohn, J. 2003) as dermal scaffolds for making HSE with improved mechanical strength and handling.

2. MATERIALS AND METHODS

2.1 Establishment of Cell Lines

Primary keratinocytes and dermal fibroblasts were obtained from Invitrogen (Carlsbad, CA). The fibroblasts were grown in Dulbecco's Modified Eagles Medium (DMEM) (Gibco-Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and passages 3 to 10 were used for the skin equivalent preparation. Keratinocytes were cultured in supplemented Epilife (Invitrogen, Carlsbad, CA). Keratinocytes passages 2 or 3 were used for the preparation of the skin equivalents.

2.2 Preparation of Full Thickness Skin Equivalents

A collagen based dermal matrix was prepared by mixing together 2X DMEM (Invitrogen Corp., Carlsbad, CA), FBS (Atlanta Biologicals, Lawrenceville, GA), 0.1 N NaOH (Sigma, St. Louis, MO), bovine type I collagen (Inamed, Santa Barbara, CA), and 1 ml of a suspension of fibroblasts at a concentration of 220,000 cells/ml in a 60 mm petri dish. Over 7 days of incubation at 10% CO_2, 37 °C the collagen was contracted by the cells into a tissue and subsequently keratinocytes (~700,000 cells/layer) were seeded on top of the dermal layers. After 1.5 h of attachment, the skin equivalents were cultured submerged in media for 7 days to allow proliferation of keratinocytes and the development of the basal layer of epidermis. On the 7th day, the cultures were lifted to the Air-Liquid interface (ALI) and fed only from the bottom. Culture at the ALI for 21 days allowed full differentiation of keratinocytes leading to formation of a differentiated, multilayered, full thickness SE.

During culture of the HSE, the media was supplemented with external factors in order to obtain a fully differentiated skin model that depicts the biochemical properties of human skin. A mixture of external fatty acids (25 µM palmitic acid, 15 µM linoleic acid, 25 µM oleic acid, 7 µM arachidonic acid), ascorbic acid (100 µg/ml) and a Peroxisome Proliferator Activated Receptor (PPAR) agonist clofibrate (300 µM) was added to the media during culture at the ALI.

2.3 Morphology, Lipid Composition and permeability studies.

Morphological analysis was conducted by staining 5µm paraffin embedded sections with Hematoxylin and Eosin (H & E) and subsequent light microscopy analysis. The lipid composition was determined by thin layer chromatography. Permeability studies were conducted with the aid of vertical glass Franz diffusion cells (Permegear, Inc., Bethlehem, PA) and the permeability parameters determined for the HSE were compared to those of human cadaver skin and Epiderm FT®. The actives selected for permeability studies were the model agent caffeine, ketoprofen and hydrocortisone, the CWA mimics malathion and Paraoxon, and the insect repellant DEET (all varying in their lipophilicities). All the above chemicals were purchased from Sigma (St. Louis, MO) and analyzed using validated HPLC methods run on an Agilent HP1100 system and an Eclipse XDB-C_{18} 5 µm column (Agilent Technologies).

2.4 Synthesis of a electrospun scaffold for making HSE

A 22% wt/vol solution of the polymer poly(DTE carbonate) was made in the solvent mixture of THF/DMF and electrospun into a 3D fiber mat (Figure 1). To generate a hybrid scaffold by freeze-drying collagen on top of electrospun polymer fiber mat, 1 mg/ml of collagen solution was poured on top of the polymer mat and allowed to penetrate into the mat for 20-30 minutes. The entire setup was then frozen slowly till -40^{o}C at a rate of 1^{o}C /min, followed by lyophilization for about 20 hrs, at 0^{o}C. This method results in a porous collagen layer, on top of the electrospun polymer mat, which provides the mechanical framework. Other modifications were performed to the polymer mat to improve the cell adhesion and mechanical properties of the polymer.

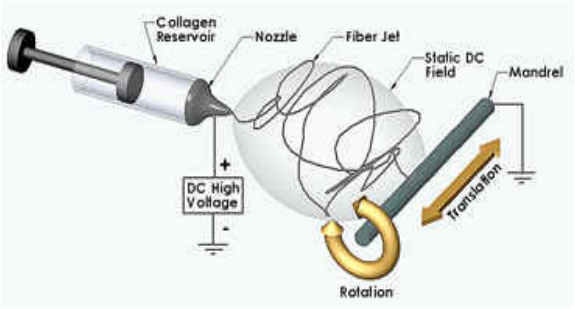


Figure 1: Schematic representation of electrospinning. Source:*http://www.people.vcu.edu/~glbowlin/electrospinning.htm*

2.5 Mechanical testing of the polymer hybrid scaffolds

About 2.5 cm X 0.6 cm strips of the polymer scaffolds was cut and mounted on to a MTS Sintech 5/D mechanical testing device attached with a 10 Newton load cell and tested. Results are shown as Young's modulus of the material (refers to stiffness) and the % strain at break (reflects the extensibility of a material).

2.6 Cell attachment and proliferation on electrospun scaffolds

Cell attachment and proliferation of human dermal fibroblasts (HDF) was measured on the improved polymer scaffold using the MTS colorimetric assay (Promega Cell Titer 96 Aqueous One Solution). For cell attachment, HDF cells were seeded to polymer scaffolds placed in 24-well plates for 24 hrs, after which scaffolds were transferred to a new well and the amounts of cells attached to scaffold was measured by the MTS assay. For cell proliferation, the cells were allowed to grow in the scaffolds for 9 days and the increase in cell population was measured on days 0, 1, 3, 5, 7 and 9. For microscopic visualization of cells populating the electrospun scaffolds, the cells were fixed with 4% paraformaldehyde (in PBS), and then stained using FM 143-FX (which stains cell membranes and intracellular vesicles) and Hoechst 33342 (stains cell nuclei) and visualized by a epifluorescence microscope (Zeiss Axio Observer D1) and a confocal/multiphoton microscope (Leica Microsystems).

2.7 Co-seeding experiments with keratinocytes & dermal fibroblast to evaluate formation of intact epidermis

Initial tracking studies of fibroblasts and keratinocytes were performed with cell-tracker red and cell tracker green at different concentrations (table below).

	Cell Tracker Green CMFDA (Conc./µM)			**Cell Tracker Red CMTPX (Conc./µM)**		
Dermal Fibroblasts	1	2.5	5.0	1	2.5	5.0
Keratinocytes	1	2.5	5.0	1	2.5	5.0

Further, co-culture studies were performed and applied on the HSE model. The collagen dermis was prepared directly in the inserts. For making the epidermis, a collagen-IV coating was applied on top of the dermal later and then about 700,000 keratinocytes were seeded to make the epidermal layer. For making the HSE using the scaffolds, the dermal layer was formed by seeding HDF cells (~250,000) in collagen on scaffolds placed directly in Transwell inserts (Corning Inc.). For forming the epidermis, Keratinocytes:Fibroblasts in ratios of :5:1 and 5:5 were seeded on top of the dermal layer. Ascorbic Acid 2-Glucoside (AA2G) (100µg/ml) was supplied during entire skin formation. Histological analysis of the skin equivalents was carried out by cryosectioning and Hematoxylin & Eosin (H&E) staining.

3. RESULTS AND DISCUSSION

3.1 Morphology, Lipid Composition and Permeability studies

When cultured without any additional growth factors (lipids, ascorbic acid and clofibrate), a full thickness HSE was obtained with a differentiated epidermis. Addition of external lipids or fatty acids has been shown to serve as precursors of barrier lipids in the SC and also induce the formation of lamellar bodies (Boyce, S. T. and Williams, M. L. 1993). Ascorbic acid has been shown to catalyze the hydroxylation reactions that are required for the formation of the essential ceramides that are key to the barrier function of the lipids in the SC (Uchida, Y., Behne, M. et al. 2001). PPAR-α receptor agonists, such as clofibrate, have been shown to normalize differentiation and accelerate epidermal barrier maturation in fetal rat skin both *in vivo* and *in vitro* (Hanley, K., Komuves, L. G. et al. 1999;Komuves, L. G., Hanley, K. et al. 1998) . When the skin equivalents were cultured with a combination of lipids, ascorbic acid (100µg/ml) and clofibrate (300 µm), the HSE exhibited relatively higher number of viable epidermal layers and a thicker stratum corneum as compared to the control. The epidermis contained all layers of native epidermis and also demonstrated plenty of keratohyalin granules in the stratum granulosum, showing a normalized differentiation pattern (Figure 2). Also, the HSE cultured with all three factors together showed a lipid profile that more closely resembled that of native human skin, as compared to the control.

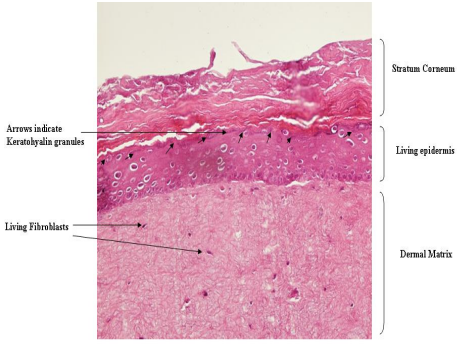


Figure 2: Light microscopy of HSE treated with lipid supplements, ascorbic acid and clofibrate

Table 1: Lipid Profile of HSE Cultures Without Any Additional Growth Factors (Control), With Clofibrate, Ascorbic Acid and Lipids Compared With the Lipid Profile of Native Human Skin

Lipid class	Control	External lipids, ascorbic acid (100 µg/ml) and clofibrate 300 µM	Human cadaver skin
Phospholipids	19.5 ± 3.8	23.8 ± 2.1	39.2 ± 2.1
Glucosylceramides	1.0 ± 0.3	5.3 ± 0.3	4.8 ± 0.7
Acylglucosylceramides	0.2 ± 0.1	0.6 ± 0.1	Trace
Ceramides	8.6 ± 1.4	14.1 ± 0.5	12.2 ± 2.2
Cholesterol	25.9 ± 3.6	25.3 ± 3.1	19.4 ± 2.9
Fatty acids	6.7 ± 0.6	10.1 ± 0.5	8.1 ± 0.6
Triglycerides	33.5 ± 3.1	15.5 ± 1.6	9.2 ± 0.9
Cholesterol esters	4.6 ± 0.8	5.3 ± 1.2	7.1 ± 0.5

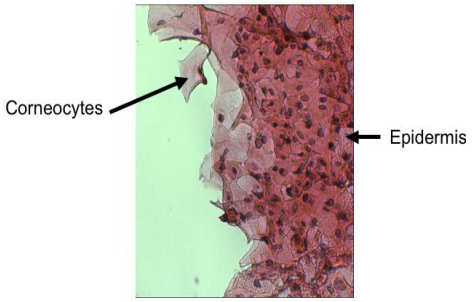


Figure 3: Horizontal section of the epidermis (bottom up) from the HSE showing the flattened corneocytes in the stratum corneum, situated above the differentiating epidermis.

Higher content of glucosylceramides and acylglucosylceramides and lower amount of triglycerides were observed as compared to control (Table 1). This lipid composition plays a crucial role in permeability since a unique lipid composition of ceramides, cholesterol and fatty acids is essential for the barrier properties of skin. The normalization of the lipid profile by a combination all three factors caused a decrease in the permeability of caffeine and hydrocortisone. While the permeability of HSE to caffeine decreased by about 76% by the addition of clofibrate, ascorbic acid and lipids, the permeability of the HSE to hydrocortisone decreased by 79% with the addition of these combination of agents (Figures 4 and 5). Overall, both reconstructed models depicted significantly higher permeability to all agents as compared to human skin (data for caffeine, DEET and malathion are depicted in Figures 6, 7 and 8). While permeabilities of caffeine, malathion and hydrocortisone were approximately 2, 3, 4 fold respectively of human skin, permeability of ketoprofen and DEET with HSE was ~ 5 and 7 times that of human skin. A high difference in the permeability of Paraoxon was observed (~25 fold higher permeability with HSE as compared to human skin), with Epiderm FT® mirroring the same permeability trend. The HSE demonstrated very similar permeation profiles for most agents when compared to Epiderm FT®.

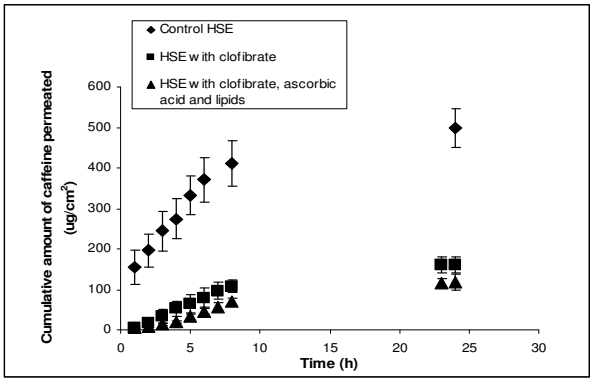


Figure 4: Amount of caffeine permeated through control and optimized HSEs

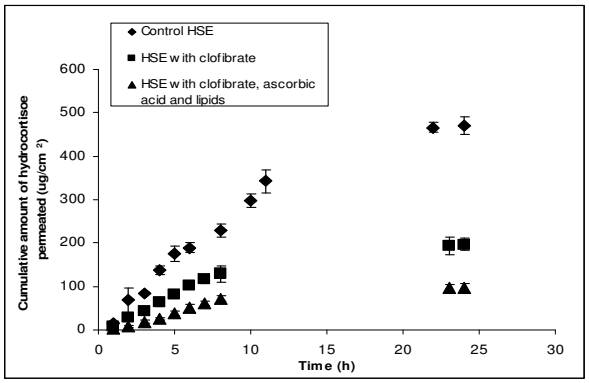


Figure 5: Amount of hydrocortisone permeated through control and optimized HSEs

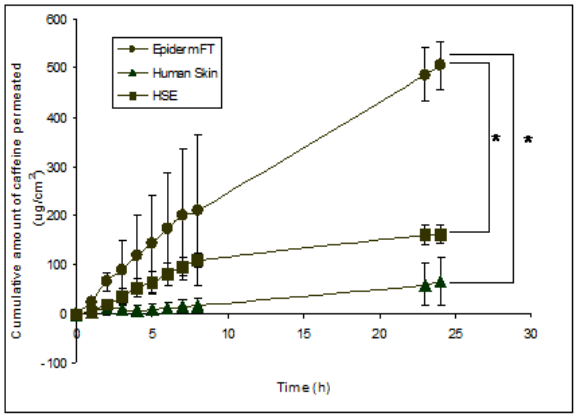


Figure 6: Permeation of caffeine through optimized HSE, Epiderm FT® and human skin

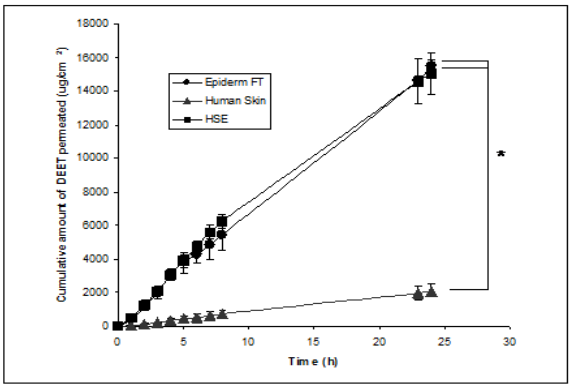


Figure 7: Permeation of DEET through optimized HSE, Epiderm FT® and human skin

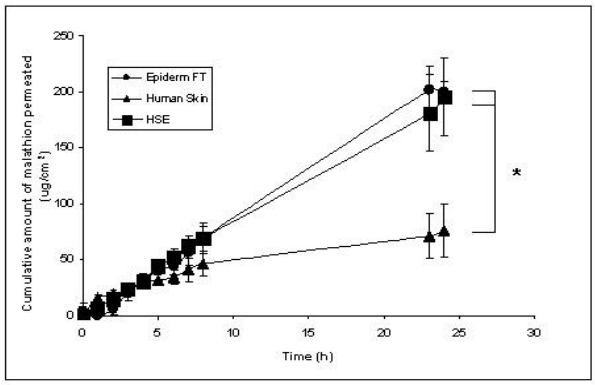


Figure 8: Permeation of malathion through optimized HSE, Epiderm FT® and human skin

However, a significant difference ($p<0.05$) was observed between HSE and Epiderm FT® for caffeine permeation, where the permeation parameters for HSE (Q_{24}: 118.56 ug/cm^2, J: 9.46 ug/cm^2/h) were significantly lower than Epiderm FT® (Q_{24}: 504.56 ug/cm^2, J: 38.53 ug/cm^2/h) ($p<0.05$). The lower permeability of HSE to caffeine, a hydrophilic low molecular weight drug caffeine, may indicate intact structures of the lipid bilayers and the corneocytes in the epidermis of the HSE which may provide a high barrier to diffusion for hydrophilic compounds. The high permeability obtained for paraoxon with the skin equivalents could be a result of an underestimation of paraoxon permeability through human skin due to its decomposition during the extensive epidermal passage of native human skin.

3.2 Electrospinning the polymer scaffold for improved mechanical and cell proliferation properties.

Lyophilization result in the generation of a porous collagen-polymer scaffold which is shown as under:

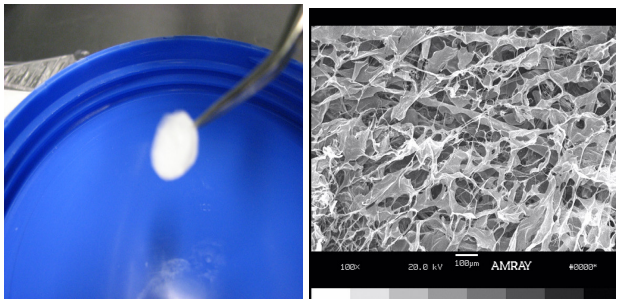
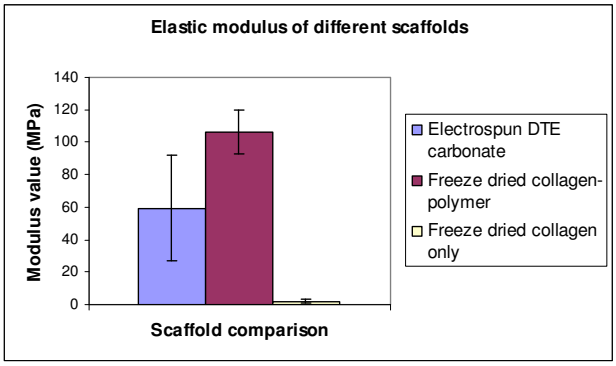
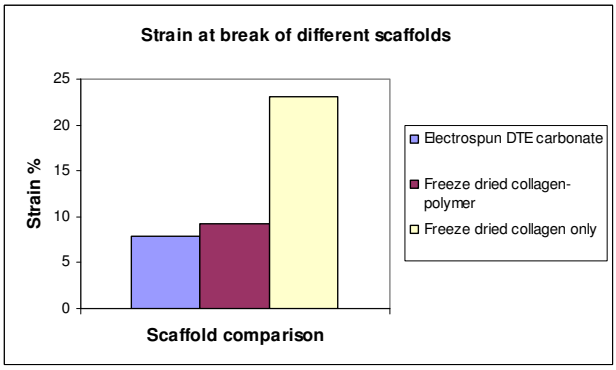


Figure 9: Collagen freeze dried onto polymer scaffold.

Introduction of collagen to the scaffold resulted in increased young modulus (representing stiffness of material) and strain at break (represent extensibility of material) for the protocol poly(DTE scaffold).



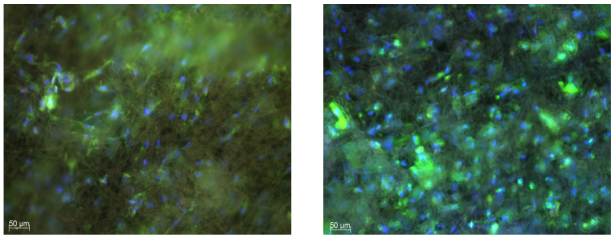
(A)



(B)

Figure 10: (A) Young's modulus of different scaffolds. (B) Strain experienced at break by different scaffolds. It was observed that collagen, due it fibrous nature does not show a fixed breakage point and extends till all the fibers get separated from each other (N=3).

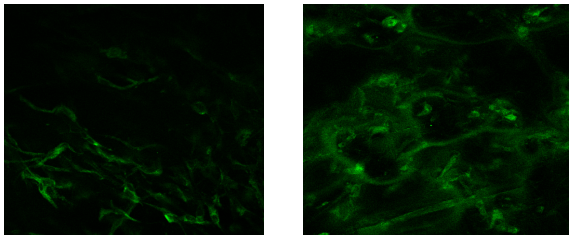
Modified polymer fiber were seeded with cells and observed by fluorescence microscopy. The results show better cell fluorescence in the improved scaffold than the poly(DTE carbonate) scaffold.



(A) Poly(DTE carbonate) scaffold (B) Improved scaffold.

Figure 11: Two color fluorescence Images of HDF cells seeded onto the electrospun scaffolds. (A) poly(DTE carbonate) scaffold & (B) Improved electrospun scaffold. Fluorescence: GREEN is FM 143-FX which stains cell membranes and intracellular vesicles; and BLUE is Hoechst for staining cell nuclei.

Results of confocal microscopy data at different depths show that the improved scaffold has more cell penetration than the poly(DTE carbonate) scaffold.



(A) poly(DTE carbonate) (B) Cells in hybrid scaffold

Figure 12: Confocal data of HDF cells growing in the electrospun scaffolds at day 9 after seeding.
(A) Cells in poly(DTE carbonate) at depth of 10 µm and (B) Cells in hybrid scaffold at depth of 10 µm.

Modification in the fiber mat composition showed improved cell adhesion properties. Results of cell attachment of human dermal fibroblasts on the improved scaffold are shown as under.

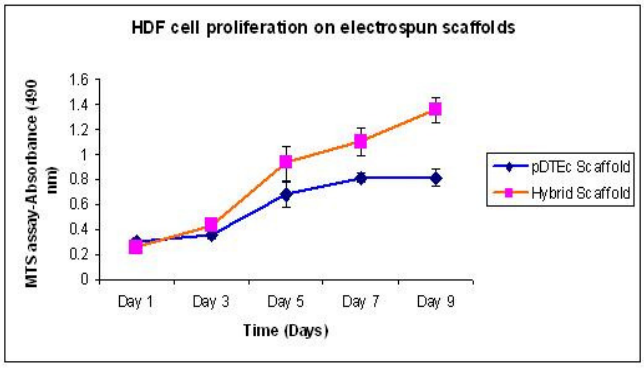
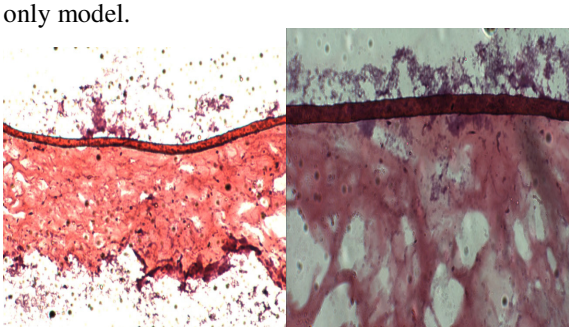


Figure 13: MTS assay carried out to quantify total cell populations in the scaffolds. (pDTEc): poly(DTE carbonate) scaffold and (Hybrid): improved polymer scaffold. N=4.

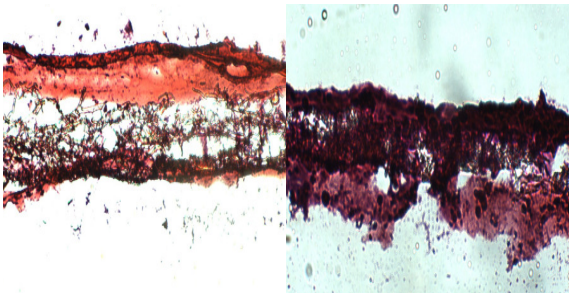
3.3 Co-culture skin cell studies for HSE

It has been observed in the past that fibroblasts play an important role in the keratinocyte differentiation (El Ghalbzouri, A., Lamme, E. et al. 2002). So, studies have been performed in the lab to evaluate the effect of fibroblast keratinocyte co-culture. For the initial studies it was observed that Cell Tracker Green CMFDA at a concentration of 5 µM seems to be the best dye to label keratinocytes, whereas Cell Tracker Red CMTPX at a concentration of 5 µM seems to be the best dye to label dermal fibroblasts for up to 5-6 days, with slight loss of viability (data not shown).

Full thickness skin was cultured with the co-culture seeding on the dermis and was compared to the collagen only model.



(A) 1X collagen FT skin. (B) 4X collagen FT skin



(C) K: F:: 5:1 (D) K:F::5:5

Fig 14: Histology of HSEs prepared using collagen only (A & B), and using poly(DTE carbonate) scaffold with Keratinocyte:Fibroblast (K:F) ::5:1 (C) and 5:5 (D) (actual cell count=× 10^5). Image (B) is a 4X magnification of (A) showing the epidermis. Image (D) is 2X compared to Image (C). The HSE with 5:1 K:F has a similarly formed epidermal layer, while for 5:5 K:F, the entire HSE tissue was more compact (thin) and the epidermal layer was very prominent. The amount of dermal component was also less in 5:5 K:F, than in 5:1 K:F. All sections above stained with H & E.

CONCLUSION

The optimization of the barrier properties of the collagen based HSE by modification of the culture media resulted in lower permeabilities for model agents. In general, the bioengineered skin equivalents demonstrated lower standard deviations as compared to human cadaver skin. Also, the HSE compared well with Epiderm FT® in terms of permeability. Though we used compounds of differing lipophilicities (including chemical warfare agent mimics) for permeability testing, it was not possible to come to a conclusion regarding the permeability behavior of these skin models to various agents, especially the differing permeabilities obtained for caffeine. Such conclusions would have to include further validation of a larger library of compounds. The development, optimization and testing of the collagen based HSE, along with its validation represents an important step toward the use of these skin equivalents as reproducible and accessible skin models for permeation testing.

A synthetic polymer containing fibrous scaffold has been created that uses a novel architecture by incorporating electrospun and freeze-dried collagen in 3 separate layers. The polymer containing scaffolds with a layer of freeze-dried collagen on top performed better in mechanical properties (stiffness and extensibility), while the hybrid scaffolds (containing electrospun polymer + collagen together) showed better penetration and proliferation for the dermal fibroblast cells, that would give rise to a better populated dermis in the HSE. Using keratinocytes: fibroblasts co-cultures in a ratio of 5:1 formed better and compact epidermal layers. Since the epidermal layer was better integrated to the dermis, it may result in better tissue (HSE) formation and probably, better epidermal barrier properties once some more culture conditions (formation of stratum corneum, etc) are standardized. The polymer integrated HSE model can offer better mechanical properties and handling compared to other known skin equivalents.

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